Ischemia alters the electrical activity of pacemaker cells isolated from the rabbit sinoatrial node

O. GRYSCHENKO, J. QU, AND R. D. NATHAN
Department of Physiology, Texas Tech University Health Sciences Center, Lubbock, Texas 79430

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Gryschenko, O., J. Qu, and R. D. Nathan. Ischemia alters the electrical activity of pacemaker cells isolated from the rabbit sinoatrial node. Am J Physiol Heart Circ Physiol 282: H2284–H2295, 2002. First published February 7, 2002; 10.1152/ajpheart.00833.2001.—The purpose of this study was to investigate the mechanisms responsible for ischemia-induced changes in spontaneous electrical activity. An ischemic-like Tyrode solution (pH 6.6) reversibly depolarized the maximum diastolic potential (MDP) and reduced the action potential (AP) overshoot (OS). We used SNARF-1, which is an indicator of intracellular pH (pHi), and perforated-patch techniques to test the hypothesis that acidosis caused these effects. Acidic but otherwise normal Tyrode solution (pH 6.8) produced similar effects. Basic Tyrode solution (pH 8.5) hyperpolarized the MDP, shortened the AP, and slowed the firing rate. In the presence of “ischemic” Tyrode solution, hyperpolarizing current restored the MDP and OS to control values. HOE-642, an inhibitor of Na/H exchange, did not alter pHi or electrical activity and did not prevent the effects of ischemic Tyrode solution or recovery after washout. Time-independent net inward current but not hyperpolarization-activated inward current was enhanced by ischemic Tyrode solution or by 30 μM BaCl2, a selective blocker of inward-rectifying K currents at this concentration. The results suggest that 1) acidosis was responsible for the ischemia-induced effects but Na/H exchange was not involved, 2) the OS was reduced because of depolarization-induced inactivation of inward currents that generate the AP upstroke, and 3) reduction of an inward-rectifying outward K current contributed to the depolarization.

AN ESTIMATED 70–80% of all electronic pacemakers (~500,000 in the US as of 1999) are implanted in patients 65 years of age and older (16). Abnormalities of sinoatrial (SA) node impulse generation as well as conduction disturbances are common in these patients and constitute much of the need for permanent pacemakers. For example, disruption of the blood supply of the SA node (ischemia) is responsible for some arrhythmias that occur shortly after orthotopic heart transplants and inferior wall acute myocardial infarctions (1, 35). Several multicellular models have been used to investigate the mechanisms responsible for ischemia-induced arrhythmias in the SA node. For example, occlusion of the SA node artery markedly slowed the firing rate of blood-perfused canine right atrial preparations (20). Similar effects were seen in rat hearts just after Langendorff perfusion was interrupted (2). In rabbit right atrial preparations, exposure to hypoxia reduced the concentrations of ATP and creatine phosphate (45), depolarized the maximum diastolic potential (MDP), and decreased the action potential (AP) overshoot (OS) and upstroke velocity as well as the slope of diastolic depolarization (25, 31, 45). Removal of glucose from the bathing solution potentiated these effects (31), and metabolic inhibitors such as cyanide and 2,4-dinitrophenol produced similar but more rapid effects (25).

In comparison with right atrial preparations, isolated SA node pacemaker cells have both advantages and disadvantages. One advantage is that electrophysiological and fluorescence techniques can be employed simultaneously to correlate changes in electrical activity with the loss or gain of intracellular ions such as Ca2+ and H+. Another advantage is that whole-cell patch-clamp techniques can be used to investigate the changes in ionic currents that underlie ischemia-induced alterations of spontaneous electrical activity. On the other hand, the disadvantages include the absence of a restricted extracellular space surrounding the cells where metabolites can accumulate and the absence of other cell types such as neutrophils, which release oxygen radicals. Despite these disadvantages, Han and coworkers (18) observed marked reductions of the amplitude, duration, and frequency of spontaneous APs when rabbit isolated SA node cells were exposed to cyanide or 2,4-dinitrophenol for 5–10 min. Even these brief exposures activated ATP- and glibenclamide-sensitive K channels (IATP) (19) and reduced L-type Ca currents (ICaL), delayed-rectifier K currents (IK), and hyperpolarization-activated inward current (Ih) (18).

During acute myocardial ischemia, the extracellular environment is characterized by hypoxia, acidosis, and increased levels of K+ (for reviews, see Refs. 9 and 10). In preliminary experiments, we observed a gradual depolarization of the membrane potential and slowing of the firing rate when rabbit isolated SA node pace-
maker cells were exposed to a glucose-free hypoxic Tyrode solution (34). Because these effects were accelerated by the reduction of extracellular pH (pH_e) to 6.8, and because a pH_e of 6.5 had altered the electrical activity of rabbit right atrial preparations in a similar fashion (37), we decided to test the hypothesis that acidosis was responsible for the “ischemia”-induced alterations we had observed previously. In the present study, we exposed SA node pacemaker cells to a glucose-free bathing solution that was titrated to pH 6.6 and bubbled with 100% N₂. A pH of 6.6 was chosen because Yan and Kleber (44) measured a pH of 6.6 in the perfusate of rabbit papillary muscle after no-flow ischemia. Intracellular pH (pH_i) is also important because it influences the rate of anaerobic glycolysis, the development of active tension, and the functions of ion channels and exchangers (9, 44). Therefore, we used SNARF-1, a fluorescent indicator of pH_i (3, 8), and perforated-patch recordings of spontaneous electrical activity to test our hypothesis.

MATERIALS AND METHODS

Isolation of SA node pacemaker cells. As approved by this institution’s Animal Care and Use Committee, male New Zealand White rabbits (body wt 1.0–1.5 kg) were stunned by a blow to the junction of the head and neck, and the heart was rapidly removed. A small hole was cut in the right atrium and infused with a HEPES-buffered salt solution (HBSS), which contained 20 mM 2,3-butanedione monoxime (BDM; Sigma-Aldrich Chemicals) to remove blood from the right atrium and eliminate its contraction (24, 39). After its excision, the right atrium was pinned to the bottom of a Sylgard-coated petri dish and immersed in fresh HBSS with BDM. The entire SA node was removed, trimmed of pericardium and fat, and cut into several pieces. Single cells were isolated as described previously (30) but with the following modifications. The pieces were digested at 37°C during 4–6 exposures (5–10 min each) to the solutions as follows: 1) 5 ml of a nominally Ca- and Mg-free buffer containing protease (P-8038, 2.8 U/ml; Sigma) and 0.1% BSA (A-2153, Sigma); 2) same as solution 1, but the enzyme buffer contained 20 μM CaCl₂ and was stirred at 200 rpm; 3) same as solution 2, but the enzyme buffer contained 30 μM CaCl₂ and the protease was replaced by type II collagenase (122–243 U/ml; Worthington Biochemicals); 4) same as solution 3, but with 50 μM CaCl₂; 5) and 6) same as step 4 if necessary. BDM (20 mM) was included sometimes with one or both of the enzymes to prevent contracture of the isolated pacemaker cells (24, 39).

Its effects on contraction and spontaneous electrical activity were reversed completely after washout and by the time the cells were studied. After each of steps 3–6, freed cells were transferred to a centrifuge tube containing either HBSS and 1.0% BSA at room temperature or a cold modified Kraftr-Bruhe (KB) solution. Both solutions also contained a mixture of protease inhibitors (P-2714; Sigma). After centrifugation (180 × g for 10 min at room temperature), the cells were resuspended in culture medium or maintained in cold KB solution for 45 min and then plated on small pieces of no. 0 glass (~3 × 3 mm and coated with laminin; L-2020; Sigma) in 35-mm plastic petri dishes. “Freshly isolated” cells were kept in KB at 4°C for up to 24 h, and “cultured” cells were maintained in an incubator (95% air-5% CO₂) at 37°C for up to 4 days.

Electrophysiology. A perforated-patch technique (22) was employed to record spontaneous electrical activity or ionic currents in freshly isolated or cultured pacemaker cells. The patch pipette contained (in mM) 75 K₂SO₄, 55 KCl, 7 MgCl₂, and 10 HEPES; and 300 μg/ml nystatin. pH was adjusted to 7.2 with KOH. A small amount of this solution without nystatin was drawn into the tip of the pipette just before a gigahm seal was made with the cell membrane. Even though monovalent cations and anions are permeable through nystatin-induced channels, divalent ions are not; therefore, K₂SO₄ was included in the pipette solution to minimize the development of a Donnan potential due to impermeant anions in the cytoplasm (22). Because this technique prevents washout of important molecules such as cAMP, “rundown” of spontaneous electrical activity in rabbit SA node pacemaker cells can be avoided (30). This was verified in the present study because the changes in electrical activity were completely reversible after washout of the ischemic Tyrode solutions. The pipette resistance was ~5 MΩ, and electronic compensation was used to minimize the series resistance that remained after membrane perforation was complete. We included whole cell currents only if the membrane potential was well controlled during their acquisition. Pacemaker cells were superfused with normal Tyrode solution containing 10 mM HEPES (Table 1). HEPES, rather than NaHCO₃/CO₂, was better able to hold the pH constant over the course of each experiment. Experiments were performed at 35 ± 1.0°C (model TC-1 temperature controller; Cell MicroControls, Virginia Beach, VA). In most experiments, membrane potentials were corrected by ~2.7 mV for the calculated pipette-to-bath liquid-junction potential. After obtaining a pipette-to-membrane seal, we waited 10–20 min for patch perforation before recording the spontaneous electrical activity. Complete exchange of superfusion solutions required 1–2 min.

Measurements of pH_i. A xenon arc lamp and a Zeiss inverted microscope provided 485-nm excitation, and a filter-based photometer system (Photon Technology International) allowed simultaneous acquisition of SNARF-1 fluorescence at 580 nm (F580) and 640 nm (F640). A shutter limited the duration of illumination to 1 s, thereby minimizing photobleaching of the dye and damage to the cells (3). Light collected by the photomultipliers was restricted by an adjustable mask to an area the size of the cell. Pacemaker cells were incubated with the acetoxymethyl ester (AM) form of SNARF-1 (2.5 μM) at room temperature for only 15 min to minimize its entry into cytoplasmic compartments (5). The cells were then superfused with normal Tyrode solution for 15–30 min to eliminate extracellular dye. Background F580 and F640, which were collected from a cell-free area the same size as the cell, were <1% of the SNARF-1 fluorescence.

Table 1. Experimental solutions

<table>
<thead>
<tr>
<th>Tyrode Solution</th>
<th>Glucose, mM</th>
<th>Bubbled with 100% Air or Na⁺</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal†</td>
<td>5.5</td>
<td>Air</td>
<td>7.4</td>
</tr>
<tr>
<td>pH 6.8</td>
<td>5.5</td>
<td>Air</td>
<td>6.8</td>
</tr>
<tr>
<td>pH 8.5</td>
<td>5.5</td>
<td>Air</td>
<td>8.5</td>
</tr>
<tr>
<td>pH 7.4 ischemic</td>
<td>0</td>
<td>N₂</td>
<td>7.4</td>
</tr>
<tr>
<td>pH 6.6 ischemic</td>
<td>0</td>
<td>N₂</td>
<td>6.6</td>
</tr>
</tbody>
</table>

*When solutions were bubbled with 100% N₂, P₀ measured in cell chamber averaged only 8% below ambient level. †Normal Tyrode solution contained (in mM) 130 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.6 MgCl₂, 0.6 NaHPO₄, 1.0 or 0 NaHCO₃, 5.5 glucose, and 10 HEPES; pH was titrated to 7.4 with NaOH.
therefore, they were neglected in calculations of the F580/F640 ratio. Compared with SNARF-1-loaded cells, the autofluorescence of unloaded cells was ~1% of F580 and <1% of F640; therefore, it too was neglected in calculations of the F580/F640 ratio.

**In situ calibration of SNARF-1 fluorescence.** Cells were loaded with 2.5 µM SNARF-1 at room temperature for 15 min and then rinsed in Tyrode solution for at least 15 min. After the F580/F640 ratio had stabilized in normal Tyrode solution, the cells were exposed to several high-K concentration (K) buffers at 35°C. Each buffer contained (in mM) 125 KCl, 5 NaCl, 1.1 MgCl2, 2.7 CaCl2, 5.0 EGTA, 10 MES [acidic dissociation constant (pK_a) = 6.1 at 25°C], 10 HEPES (pK_a = 7.5), and 10 Na,N-bis(2-hydroxyethyl)glycine (bicine, pK_a = 8.3); pH was titrated with 6 M KOH. Nigericin (9.6 µM) and valinomycin (6.4 µM) were also included to collapse the ionic gradients for K^+ and H^+ (5, 28). They were dissolved in 100% EtOH, prepared as 33.5 and 22.5 mM stock solutions, respectively, and then aliquoted and frozen until the day of the experiment. The final concentration of EtOH in each high-K^+ buffer was 0.6%. We were careful to rinse the cell chamber and tubing with EtOH and water after each experiment to remove any remaining trace levels of nigericin or valinomycin (4). Several pacemaker cells were exposed consecutively to seven high-K^+ buffers with pH ranging from 5.86 to 8.75 (Fig. 1A). At the end of this procedure, a repeat measurement of the F580/F640 ratio at pH 6.82 was quite similar to the one obtained 1 h earlier, which confirms the stability of the cells. The mean values ± SE for eight cells were plotted versus pH and fit by Eq. 1 (Fig. 1B). The in situ pK value for SNARF-1 was calculated using Eq. 2 (3, 5)

\[
R = (R_{\text{min}}^{10\log R_{\text{app}} + \log 10\beta} + R_{\text{max}}^{10\log R_{\text{app}} + \log 10\beta})
\]

where \(R = F_{580}/F_{640}\), \(pK_{\text{app}}\) is the apparent pK, and \(\beta = F_{640\text{max}}/F_{640\text{min}}\). The following are the best-fit parameters for Eq. 1: \(R_{\text{max}} = 6.96 ± 0.10, R_{\text{min}} = 1.04 ± 0.12, \) and \(pK_{\text{app}} = 7.46 ± 0.04\). Using \(pK_{\text{app}}\), the average \(\beta (1.31 ± 0.16)\), and Eq. 2, we obtained a pK of 7.58 ± 0.04. This value is consistent with other SNARF-1 pK values measured in situ: 7.4–7.6 for lens epithelial cells (3), 7.6–7.8 for rat cardiac myocytes (5). Values for the F580/F640 ratio and the best-fit parameters above were employed in Eq. 3 to calculate the pH in each of the experiments described in the results.

\[
pH = pK_{\text{app}} + \log_{10}(R_{\text{max}} - R)/(R - R_{\text{min}})
\]  

**Solutions.** The compositions of the cell-isolation solutions, culture medium, and HBSS have been described (30). The modified KB solution contained (in mM) 70 L-glutamic acid, 25 KCl, 10 KH2PO4, 3 MgCl2, 20 taurine, 10 dextrose, 0.3 EGTA, and 10 HEPES, and the pH was titrated to 7.4 with KOH. SNARF-1-AM (50 µg; Molecular Probes) was dissolved in 10 µl of anhydrous dimethylsulfoxide (DMSO; Sigma), 10 µl of Pluronic F-127 (25% wt/wt in anhydrous DMSO), and 17.6 ml of normal Tyrode solution (see Table 1). Aliquots (0.5 ml) of this solution were frozen at −80°C, thawed just before use, and added to 0.5 ml of Tyrode solution to yield a final concentration of 2.5 µM SNARF-1-AM. Table 1 lists the solutions used in the various experiments. HOE-642 (cariporide) was a gift of Aventis Pharma in Frankfurt, Germany.

**Data analysis.** Analog data were digitized at 12- or 16-bit resolution using Labmaster DMA boards, which were controlled by Felix (Photon Technology International) and pClamp 8.0 (Axon Instruments) software. For measurements of pH, Felix was employed to acquire SNARF-1 at F580 and F640 at a rate of 200 points/s and to average the raw data over 1-s periods. Periods of 10–20 s were used by pClamp to acquire spontaneous electrical activity. The MDP, OS, duration (Dur) at −20 mV, and frequency of APs (beat rate, BR) were measured from several APs and averaged. These parameters are presented as means ± SE for those cells exposed to a particular condition. Two-tailed paired Student’s t-tests were used for statistical analyses. Differences between means were considered significant if \(P < 0.05\).
confirm the identity of pacemaker cells. Although data were collected from pacemaker cells that had been in culture for 1–4 days, 2- and 3-day cells were employed most often because these cells provided the most consistent results. Nevertheless, there were no significant differences among the results obtained from 1-, 2-, 3-, and 4-day cells. For example, the pH$_i$ values among 2-day cells (7.24 ± 0.03, n = 37), 3-day cells (7.26 ± 0.04, n = 35), and 4-day cells (7.33 ± 0.07, n = 14) did not differ significantly.

**pH 6.6 ischemic Tyrode solution.** The purpose of these experiments was to simulate ischemic conditions in vivo. Nevertheless, even though myocardial ischemia is characterized by increased levels of K$^+$ (9, 10), we decided not to include elevated [K] in the pH 6.6 ischemic Tyrode solution (see Table 1), because the strong K depolarization of the MDP would mask the unknown effects of the remaining components (reduced pH, hypoxia, and the absence of glucose). For example, normal Tyrode solution that contained 10–13 mM KCl depolarized the MDP by 17 ± 4 mV (range = 11–24 mV, n = 4). Figure 2A illustrates the effects of pH 6.6 ischemic Tyrode solution on spontaneous electrical activity. After only 3 min, the MDP had depolarized by 14 mV, the OS had fallen by 3 mV, and the Dur had shortened by 9 ms; there was no change in BR. As shown in Fig. 2B, there was essentially complete recovery during washout. Of the twelve pacemaker cells exposed to pH 6.6 ischemic Tyrode solution for up to 4 min, twelve exhibited depolarization of the MDP and nine exhibited reduction of the OS. On average, the MDP depolarized from −65 ± 1 to −53 ± 2 mV (P < 0.00001), and the OS declined from 24 ± 2 to 20 ± 2 mV (P < 0.05). The Dur and BR were not altered significantly (Table 2), and both the MDP and OS recovered completely during washout of pH 6.6 ischemic Tyrode solution (data not shown). In the cell shown

<table>
<thead>
<tr>
<th>Condition</th>
<th>MDP, mV</th>
<th>OS, mV</th>
<th>Dur, ms</th>
<th>Mean Rate, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 12)</td>
<td>−65 ± 1</td>
<td>24 ± 2</td>
<td>243 ± 25</td>
<td>59 ± 6</td>
</tr>
<tr>
<td>pH 6.6 ischemic Tyrode solution</td>
<td>−53 ± 2</td>
<td>20 ± 2</td>
<td>258 ± 18</td>
<td>58 ± 6</td>
</tr>
<tr>
<td>Control (n = 10)</td>
<td>−67 ± 2</td>
<td>20 ± 3</td>
<td>230 ± 21</td>
<td>65 ± 3</td>
</tr>
<tr>
<td>pH 7.4 ischemic Tyrode solution</td>
<td>−66 ± 2</td>
<td>20 ± 3</td>
<td>224 ± 29</td>
<td>60 ± 3</td>
</tr>
<tr>
<td>Control (n = 10)</td>
<td>−63 ± 2</td>
<td>22 ± 2</td>
<td>305 ± 43</td>
<td>61 ± 4</td>
</tr>
<tr>
<td>pH 6.8 Tyrode solution</td>
<td>−52 ± 3</td>
<td>27 ± 3</td>
<td>336 ± 38</td>
<td>61 ± 6</td>
</tr>
<tr>
<td>Control (n = 7)</td>
<td>−64 ± 2</td>
<td>25 ± 1</td>
<td>188 ± 16</td>
<td>81 ± 3</td>
</tr>
<tr>
<td>pH 8.5 Tyrode solution</td>
<td>−69 ± 2</td>
<td>25 ± 1</td>
<td>177 ± 16</td>
<td>78 ± 3</td>
</tr>
<tr>
<td>Control (n = 7)</td>
<td>−70 ± 3</td>
<td>25 ± 2</td>
<td>190 ± 18</td>
<td>68 ± 6</td>
</tr>
<tr>
<td>5–10 mM NH$_4$Cl</td>
<td>−65 ± 3</td>
<td>25 ± 2</td>
<td>202 ± 19</td>
<td>67 ± 6</td>
</tr>
<tr>
<td>Control (n = 8)</td>
<td>−63 ± 2</td>
<td>26 ± 2</td>
<td>209 ± 11</td>
<td>71 ± 6</td>
</tr>
<tr>
<td>pH 6.6 ischemic Tyrode solution + HOE-642</td>
<td>−54 ± 2</td>
<td>24 ± 2</td>
<td>227 ± 14</td>
<td>67 ± 5</td>
</tr>
<tr>
<td>Control (n = 4)</td>
<td>−65 ± 4</td>
<td>28 ± 5</td>
<td>197 ± 15</td>
<td>59 ± 8</td>
</tr>
<tr>
<td>Normal Tyrode solution + 30 μM HOE-642</td>
<td>−64 ± 4</td>
<td>29 ± 5</td>
<td>206 ± 20</td>
<td>63 ± 4</td>
</tr>
<tr>
<td>Control (n = 7)</td>
<td>−62 ± 1</td>
<td>26 ± 2</td>
<td>211 ± 18</td>
<td>66 ± 5</td>
</tr>
<tr>
<td>pH 6.6 ischemic Tyrode solution</td>
<td>−52 ± 1</td>
<td>22 ± 2</td>
<td>212 ± 20</td>
<td>73 ± 7</td>
</tr>
<tr>
<td>pH 6.6 ischemic Tyrode solution + injection of hyperpolarizing current</td>
<td>−61 ± 1</td>
<td>27 ± 1</td>
<td>213 ± 16</td>
<td>44 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of freshly isolated and/or cultured pacemaker cells. *Membrane potentials were corrected by −2.7 mV to account for calculated liquid-junction potential between patch pipette and bath. MDP, maximum diastolic potential; OS, action potential overshoot; Dur, action potential duration measured at −20 mV. A two-tailed Student's t-test for paired variants was used to determine whether differences in mean values were statistically significant (P < 0.05); †P < 0.05, ‡P < 0.001.
in Fig. 2C, pH$_i$ began to fall ~4 min after normal Tyrode solution was replaced by pH 6.6 ischemic Tyrode solution. Complete exchange of solutions was achieved in 1–2 min. A reduction of pH$_i$ was confirmed in another 7 pacemaker cells.

**Role of pH.** To explore the mechanisms responsible for ischemia-induced reductions of the MDP and OS, we tested the components of pH 6.6 ischemic Tyrode solution (see Table 1). “Hypoxic” Tyrode solution, which contained glucose (5.5 mM) but was bubbled with 100% N$_2$, reduced pH$_i$ very little and had no significant effect on the MDP, OS, Dur, or BR of 4 freshly isolated pacemaker cells (data not shown). This lack of effect was probably because the PO$_2$ of hypoxic Tyrode solution flowing through the cell chamber was only 8% below the ambient level. Figure 3A illustrates the effects of ischemic Tyrode solution with normal pH$_o$ (7.4). Although the Dur decreased from 336 to 313 ms and the MDP depolarized by 2 mV in this cell, the changes in Dur, MDP, OS, and BR for 10 pacemaker cells were not significant (Table 2). As indicated in Fig. 3C, the pH$_i$ fell slightly in 4 of 7 cells, but it did not change in the other 3. Unlike pH 7.4 ischemic Tyrode solution, the reduction of pH of normal Tyrode solution from 7.4 to 6.8 did depolarize the MDP and attenuate the OS significantly. Of 10 pacemaker cells, 10 exhibited depolarization of the MDP and 8 exhibited reduction of the OS. On average, the MDP depolarized from −63 ± 2 to −52 ± 3 mV (P < 0.00001), and the OS declined from 32 ± 2 to 27 ± 3 mV (P < 0.05). In the example illustrated in Fig. 3B, the MDP depolarized from −69 to −60 mV, but there was no change in OS. An increase in Dur, which was only 9% in this experiment, was observed in 8 of 10 pacemaker cells. Nevertheless, on average the Dur values for normal Tyrode solution (305 ± 43 ms) and pH 6.8 Tyrode solution (336 ± 38 ms) were not significantly different. In 8 cells exposed to pH 6.6 Tyrode solution, however, the increase in Dur (from 248 ± 24 to 284 ± 34 ms) was significant (P < 0.05). Figure 3C illustrates the relationship between the MDP and pH$_i$. When this pacemaker cell was exposed to pH 7.4 ischemic Tyrode solution, pH$_i$ decreased little (from 7.38 to 7.34) and there was no significant change in the MDP. In contrast, when the same cell was exposed to normal Tyrode solution titrated to pH 6.8, the MDP depolarized 11 mV in parallel with a decline in pH$_i$ from 7.36 to 7.20. Similar reductions of pH$_i$ were seen in another 4 cells. Taken together, these results suggest that the significant depolarization of the MDP and decline of the OS induced by pH 6.6 ischemic Tyrode solution (Table 2) resulted from the acidic pH of the solution and not from hypoxia or the absence of glucose.

To further explore the role of pH, we exposed pacemaker cells to otherwise normal Tyrode solution but with the pH titrated to 8.5. In the example illustrated in Fig. 4A, the MDP hyperpolarized (from −68 to −71 mV), the Dur was shortened by 5%, and the BR was slowed by 5%; there was no change in the OS. Recovery was essentially complete after washout of pH 8.5 Tyrode solution (Fig. 4B). Of the 7 pacemaker cells exposed to pH 8.5 Tyrode solution, 7 exhibited hyperpolarization of the MDP, 6 exhibited shortening of the Dur, and 5 exhibited slowing of the BR. On average, the MDP hyperpolarized from −64 ± 2 to −69 ± 2 mV (P < 0.0001), the Dur decreased from 188 ± 16 to 177 ± 16 ms (P < 0.05), and the BR slowed from 81 ± 3 to 78 ± 3 beats/min (P < 0.05); there was no change in the OS (Table 2). The slower BR could have been due to the longer time to reach threshold from a hyperpolarized MDP. An increase in pH$_i$ after extracellular alkalosis (Fig. 4C) was confirmed in another 6 pacemaker cells. These results and those obtained with pH 6.8 Tyrode

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**Fig. 3.** Role of pH in the changes produced by pH 6.6 ischemic Tyrode solution. A: a 2-day pacemaker cell was exposed to pH 7.4 ischemic Tyrode solution for 10 min. B: a 3-day pacemaker cell was exposed to otherwise normal Tyrode solution but with pH titrated to 6.8 for 4 min. C: in the same cell, the MDP depolarized and repolarized almost in parallel with the fall and partial recovery of pH$_i$.**
solution support the hypothesis that pH alone can modulate spontaneous electrical activity.

To investigate the role of pHi with pHo fixed at 7.4, we briefly (2–4 min) exposed pacemaker cells to NH₄Cl. This common protocol first increases pHi as NH₃ enters the cells and then decreases pHi during washout of NH₄Cl as long as NH₄⁺ remains in the cytoplasm (6). The results of these experiments were unexpected: the MDP depolarized not while pHi was falling but when pHi was rising. In the example illustrated in Fig. 5A, during alkalosis the MDP depolarized from −76 to −66 mV, the OS increased from 28 to 29 mV, the Dur increased by 8%, and the BR increased by 7%. Recovery was almost complete after washout of NH₄Cl (Fig. 5B). On average, during alkalosis the MDP depolarized from −70 ± 3 to −65 ± 3 mV (n = 7; P < 0.001). In contrast, there was no significant change in OS, Dur, or BR (Table 2). In 3 simultaneous recordings of SNARF-1 fluorescence and electrical activity, the MDP depolarized in parallel with alkalosis; however, in another 3 cells, the depolarization preceded the increase in pHi as in Fig. 5C. Recovery of the MDP occurred during acidosis in 3 of the cells (Fig. 5C), but the MDP did not recover in the other 3. In contrast, all 7 of the cells reported in Table 2 exhibited recovery of the MDP after washout of NH₄Cl. A mechanism for...
from acidosis (calculated as buffering power reduced from 7.5 to 6.5, net acid efflux during recovery). Jones and co-workers (41) found that when pH o was exposed for 8 min to normal Tyrode solution containing 30 mM NH4Cl in normal Tyrode solution; this increased the time constant for recovery (τ) from 1.7 to 7.1 min. B: in another pacemaker cell, exposure to 10 μM HOE-642 in normal Tyrode solution increased τ from 5.1 to 10.9 min. Origin software (OriginLab) was used to fit the time course of recovery by a single exponential.

NH4Cl-induced changes in the MDP is described in the DISCUSSION.

Role of Na/H exchange. It has been suggested that ischemia-induced intracellular acidification activates Na/H exchange and leads to increased intracellular Na (Na i) and then to intracellular Ca (Ca i) overload as a result of reduced Na/Ca exchange (38). More recent studies, however, have come to a different conclusion (36). Because pH was an important factor in the ischemia-induced changes in electrical activity described here, we tested the hypothesis that Na/H exchange also played a role. Previous studies have shown that extracellular acidosis attenuates Na/H exchange, thereby resulting in a reduction of pH i. For example, Vaughan-Jones and co-workers (41) found that when pH o was reduced from 7.5 to 6.5, net acid efflux during recovery from acidosis [calculated as buffering power × d(pH i)/dt] was reduced by 65%. We observed qualitatively similar effects in SA node pacemaker cells. For example, the recovery of pH i after NH4Cl-induced acidosis was slowed markedly when a cell was exposed to pH 6.6 Tyrode solution: the time constant for recovery increased from 1.7 to 7.1 min (Fig. 6A). On average, the time constant for recovery increased from 3.2 ± 0.7 to 8.2 ± 1.6 min (n = 8; P < 0.005). To complement these experiments, we used HOE-642, a potent and selective inhibitor of the Na/H exchanger isoform 1 (NHE-1; see Ref. 36), to test the hypothesis that the reduction of pH i and concomitant depolarization of the MDP induced by pH 6.6 ischemic Tyrode solution were due to inhibition of Na/H exchange. At a concentration of 30 μM, HOE-642 blocked the recovery of pH i in 2 cells after NH4Cl-induced acidosis, which confirms that, like amiloride (7), HOE-642 can block Na/H exchange in rabbit SA node pacemaker cells. At a concentration of 10 μM, HOE-642 blocked the recovery of 1 cell and slowed the recovery of 5 others. For example, the time constant for recovery from acidosis was increased from 5.1 to 10.9 min when HOE-642 was present (Fig. 6B). On average, 10 μM HOE-642 increased this time constant from 3.2 ± 0.6 to 6.9 ± 2.0 min (n = 5; P < 0.05). However, compared with pH 6.6 ischemic Tyrode solution and pH 6.8 Tyrode solution (see Figs. 2 and 3), HOE-642 had a smaller effect on normal pH i. In the presence of pH 7.4 Tyrode solution, 30 μM HOE-642 reduced pH i by <0.05 pH unit in 4 cells and had no measurable effect in 4 others. Taken together, these results suggest that the reduction of pH i by pH 6.6 ischemic Tyrode solution (see Fig. 2) cannot be explained by blockade of Na/H exchange.

Although blockade of Na/H exchange was not responsible for the reduction of pH i, did this intracellular acidosis stimulate Na/H exchange? Such stimulation would have increased Na i and then Ca i via Na/Ca exchange. Could this enhancement of Na i and Ca i have depolarized the MDP and reduced the OS? If these ideas are correct, blockade of Na/H exchange would have prevented the changes in MDP and OS. Nevertheless, addition of HOE-642 to pH 6.6 ischemic Tyrode solution did not prevent depolarization of the MDP and reduction of the OS (Fig. 7A). In fact, the MDP changed

Fig. 7. HOE-642 does not modify spontaneous electrical activity or prevent the effects of pH 6.6 ischemic Tyrode solution or its recovery. A: a 2-day pacemaker cell was exposed for 4 min to pH 6.6 ischemic Tyrode solution containing 25 μM HOE-642. B: a 4-day pacemaker cell was exposed for 5 min to pH 6.6 ischemic Tyrode solution containing 20 μM HOE-642. Replacement of this solution by normal Tyrode solution containing 30 μM HOE-642 did not prevent recovery of the electrical activity after 4 min (compare with control). C: a 3-day pacemaker cell was exposed for 8 min to normal Tyrode solution containing 30 μM HOE-642.
from $-63 \pm 2$ to $-54 \pm 2$ mV ($P < 0.00001$), and the OS changed from $26 \pm 2$ to $24 \pm 2$ mV ($P < 0.05$) in 8 pacemaker cells. These effects were not significantly different from those produced by pH 6.6 ischemic Tyrode solution or pH 6.8 Tyrode solution (Table 2). Again, neither the Dur nor BR was changed significantly. Even though HOE-642 slowed the recovery of pH$i$ from acidosis (see Fig. 6B), the drug had no effect on recovery of the MDP and OS when pH 6.6 ischemic Tyrode solution was replaced by normal Tyrode solution containing HOE-642 (Fig. 7B). Similar results were obtained in 6 of 8 pacemaker cells. Finally, HOE-642 itself had no effect on electrical activity. As illustrated in Fig. 7C, the MDP and OS were unchanged during an 8-min exposure to 30 $\mu$M HOE-642, a concentration 30-fold greater than that used to block NHE-1 in isolated rat ventricular myocytes (36). On average, the MDP changed from $-65 \pm 4$ to $-64 \pm 4$ mV ($P > 0.05$) and the OS changed from $28 \pm 5$ to $29 \pm 5$ mV ($P > 0.05$) when 4 pacemaker cells were exposed to 30 $\mu$M HOE-642 (Table 2). Taken together, these results suggest that although pH 6.6 ischemic Tyrode solution reduced pH$i$, Na/H exchange played no role in the concomitant depolarization of the MDP and reduction of the OS.

**Role of ion channels.** In contrast to a direct effect of acidosis, reduction of the OS when pacemaker cells were exposed to pH 6.6 ischemic Tyrode solution or pH 6.8 Tyrode solution (Table 2) could have resulted indirectly from depolarization-induced inactivation of inward currents that generate the upstroke of the AP. To test this hypothesis, we injected hyperpolarizing current after pacemaker cells had been depolarized by pH 6.6 ischemic Tyrode solution. As illustrated in Fig. 8, when the MDP was forced back to the control level, the BR slowed and the OS recovered completely in 5 of 7 pacemaker cells. On average, when these cells were exposed to pH 6.6 ischemic Tyrode solution, the MDP depolarized from $-62 \pm 1$ to $-52 \pm 1$ mV ($P < 0.001$) and the OS decreased from $26 \pm 2$ to $22 \pm 2$ mV ($P < 0.05$; Table 2). While cells were exposed to pH 6.6 ischemic Tyrode solution, injection of hyperpolarizing current repolarized the MDP to $-61 \pm 1$ and increased the OS to $27 \pm 1$ mV, both of which are consistent with the control values. Hyperpolarizing current also slowed the BR significantly, from $66 \pm 5$ to $44 \pm 5$ beats/min ($P < 0.05$). We observed similar changes in 2 pacemaker cells exposed to pH 6.6 Tyrode solution.

These results support the idea that reductions of the OS, when cells were exposed to pH 6.6 ischemic Tyrode solution, were due to acidosis-induced depolarization of the membrane potential. But what caused this depolarization? One possibility is enhancement of an inward current. A small hyperpolarization-activated inward current ($I_h$) has been observed at the MDP of some cultured (27) and freshly isolated SA node pacemaker cells (46); therefore, we tested the hypothesis that $I_h$ was enhanced by our ischemic conditions. Nevertheless, with $I_h$ defined as the time-dependent inward current during hyperpolarizing voltage steps, we found no significant increase in its amplitude when cells were exposed to pH 6.6 ischemic Tyrode solution (Fig. 9, A–C). In fact, there was little difference in the current-voltage ($I$-$V$) relationships for $I_h$ (Fig. 9D). For example, mean values of $I_h$ in 9 pacemaker cells exposed to normal Tyrode solution ($-16.6 \pm 4.7$ pA) and then exposed to pH 6.6 ischemic Tyrode solution ($-17.1 \pm 3.8$ pA) did not differ significantly at $-70$ mV, a potential reached by some of the pacemaker cells. In contrast, the initial (time-independent) net inward current ($I_{initial}$), which preceded the onset of $I_h$ did increase in 7 of these cells (Fig. 9B). On average, $I_{initial}$ increased significantly at $-70$ mV from $-24 \pm 12$ to $-40 \pm 12$ pA ($P < 0.05$). This increase was not due to a gradual increase in leakage current, because $I_{initial}$ decreased during washout and after 5 min its mean ($-25 \pm 16$ pA) did not differ from the control. To investigate $I_{initial}$ further, we used additional test potentials to cover the diastolic range between $-60$ and $-40$ mV. As illustrated in Fig. 9E, pH 6.6 ischemic Tyrode solution reversibly increased $I_{initial}$ by 2–5 pA at those potentials. Similar results were obtained in another 5 pacemaker cells.

Finally, we exposed pacemaker cells to normal Tyrode solution containing 30 $\mu$M BaCl2 to investigate the nature of $I_{initial}$. Like pH 6.6 ischemic Tyrode solution, this concentration increased $I_{initial}$ by 2–5 pA in 4 pacemaker cells. Again, like pH 6.6 ischemic Tyrode solution, normal Tyrode solution containing 20–50 $\mu$M HOE-642 slowed the BR significantly, from $66 \pm 5$ to $44 \pm 5$ beats/min ($P < 0.05$). We observed similar changes in 2 pacemaker cells exposed to pH 6.6 Tyrode solution.
BaCl₂ reversibly depolarized the MDP by 11 ± 2 mV (n = 7; P < 0.001).

**DISCUSSION**

The purpose of this study was to investigate, in isolated SA node pacemaker cells, the mechanisms responsible for ischemia-induced changes in spontaneous electrical activity. The most important findings are 1) pH 6.6 ischemic Tyrode solution reversibly decreased the OS and depolarized the MDP; 2) similar changes were produced by normal Tyrode solution if the pH was titrated to ≤6.8; 3) increasing pH₀ to 8.5 hyperpolarized the MDP, shortened the Dur, and slowed the BR; 4) although HOE-642 slowed or blocked the recovery of pH after NH₄Cl-induced acidosis, this inhibitor of Na/H exchange did not prevent the changes induced by pH 6.6 ischemic Tyrode solution or the recovery during washout; 5) in the presence of pH 6.6 ischemic Tyrode solution, injection of hyperpolarizing current restored the MDP and OS to original values; 6) pH 6.6 ischemic Tyrode solution increased time-independent, net inward current (I_initial) but not Iᵢ in the pacemaker range of potentials; and 7) BaCl₂ (20–50 μM) in normal Tyrode solution reversibly increased I_initial and depolarized the MDP.

**Ischemia-induced changes in pH.** The reduction of pH₀ during ischemia is partially due to hampered removal of extracellular CO₂ and lactate when blood flow is diminished (10). For example, after 10 min of global ischemia, pH₀ was reduced to 6.54–6.66 (12) in the isolated, perfused rabbit interventricular septum and to 6.86 in blood-perfused rabbit papillary muscle (44). In the present study, we titrated the pH of ischemic Tyrode solution to 6.6 to approximate global ischemia in situ. The sources of intracellular acidosis during ischemia are controversial but most likely are due to the retention of H⁺ from glycolytic ATP turnover, CO₂ accumulation, and eventually, net ATP breakdown (14). Furthermore, extracellular acidosis inhibits Na/H exchange (41), thereby hampering the removal of excess H⁺ in the cytoplasm. Because of these multiple factors, the extent of acidosis is quite variable. For example, pHᵢ fell from 7.0 to 6.6 after only 4 min of global ischemia in rabbit hearts (29), but to only 6.89 after 12 min of global ischemia in perfused rabbit papillary muscles (44). By comparison, pHᵢ fell from 7.2 to 6.5 in isolated rat ventricular myocytes exposed to simulated ischemia for 30 min (glucose-free anoxic Tyrode solution with pH titrated to 6.4) (26). To insure complete recovery of the electrophysiological properties, we exposed isolated pacemaker cells to pH 6.6 ischemic Tyrode solution for only 4–5 min. During this period, pHᵢ fell by only 0.06–0.15 unit. Still, this brief treatment was sufficient to depolarize the MDP by 12 mV. The following are two possible explanations: 1) pHᵢ, not pH₀, was responsible for the depolarization; and 2) the changes in pHᵢ were actually much greater at the sarcolemma, where they could influence ion channels; however, we could not detect them by observing whole cell-averaged SNARF-1 fluorescence. This problem could have been particularly severe in our experiments with NH₄Cl, when pHᵢ was constantly changing and therefore even more likely to be nonuniform.

**Ischemia-induced changes in SA node electrical activity.** In the rabbit right atrium, which contains the SA node, hypoxia (Tyrode solution was bubbled with 95% N₂-5% CO₂) depolarized the MDP and reduced the
OS, AP upstroke velocity, and slope of diastolic depolarization (25, 31, 45); removal of glucose potentiated these effects (31). Metabolic inhibition with cyanide or 2,4-dinitrophenol (25) produced similar but more rapid effects.

In freshly isolated rabbit SA node pacemaker cells, 5- to 10-min exposures to cyanide or 2,4-dinitrophenol markedly reduced the amplitude, duration, and frequency of spontaneous APs and attenuated ICa,L, IK, and I(Ca) (18). Han and co-workers (19) have also shown that cyanide activates KATP channels in these cells. In contrast, we did not observe shortening of the AP when pacemaker cells were exposed to pH 6.6 ischemic Tyrode solution. Such a shortening would be expected if IKATP were activated. This discrepancy might be due in part to the much shorter duration (4–5 min) and therefore less metabolic blockade produced by pH 6.6 ischemic Tyrode solution in our experiments compared with the 60-min exposures to cyanide employed in the previous study. Another difference between our study and previous ones is that pH 6.6 ischemic Tyrode solution did not slow pacemaker activity, possibly because this treatment was too brief to activate IKATP. In comparison, Posner and co-workers (33) found that the mean BR of isolated right atria was slowed significantly from 173 to 129 beats/min when rabbits were raised in a hypoxic environment (PO2 = 65 mmHg) for 3 wk. They also showed that acidosis alone could slow BR dramatically (on average, from 173 to 18 beats/min). Nevertheless, an important difference between their experiments and ours is that they exposed the atria to pH 6.5 Tyrode solution for 1 h, whereas we exposed SA node pacemaker cells to pH 6.6 or 6.8 Tyrode solution for an average of only 4.1 ± 0.2 min (n = 18).

pH-induced changes in SA node electrical activity. There is little information about pH-induced changes in SA node electrical activity and the underlying mechanisms. In small pieces of rabbit SA node tissue, reduction of pH, to 6.5 depolarized the MDP, slowed the BR, and reduced the OS and maximum upstroke velocity, but had no effect on Dur (37). Opposite effects were seen when pH, was increased to 8.5. Some of the present results are similar: exposure of pacemaker cells to pH 6.8 Tyrode solution for 4 min produced an 11-mV depolarization of the MDP and a 5-mV reduction of the OS, but no significant effect on Dur; exposure to pH 8.5 Tyrode solution hyperpolarized the MDP by 5 mV. In contrast to the previous study, increasing pH, to 8.5 shortened the Dur and had no effect on the OS. In SA node tissue, the BR was slowed (by 6.6%) 10 min after pH, was reduced to 6.5 and BR was accelerated (by 10.1%) 10 min after pH, was increased to 8.5. In contrast, we observed no change in BR 4 min after pH, was reduced to 6.8 and a significant slowing of BR (by 3.7%) 4 min after pH, was increased to 8.5. Unfortunately, Satoh and Seyama (37) did not mention whether any of the changes they observed were statistically significant. Although some of the results of the two studies differ, this is not surprising because the extracellular environments of isolated pacemaker cells and pacemaker cells within SA node tissue are so different.

Role of Na/H exchange. Na/H exchange contributes to the maintenance of H+ homeostasis in rabbit SA node cells (7), and extracellular acidosis attenuates Na/H exchange in sheep Purkinje fibers (41). Therefore, we tested the hypothesis that pH 6.6 ischemic Tyrode solution inhibits Na/H exchange, thereby reducing pH, in SA node pacemaker cells. We found that the time constant for recovery of pH, from NH4Cl-induced acidosis was increased significantly (from 3.2 to 8.2 min) by pH 6.6 Tyrode solution, which suggests that acidic pH, does indeed attenuate Na/H exchange. Nevertheless, in the presence of pH 7.4 Tyrode solution, 30 μM HOE-642, a potent inhibitor of NHE-1 (36), which also significantly increased the time constant for recovery of pH, from NH4Cl-induced acidosis (from 3.2 to 6.9 min), reduced pH, by <0.05 pH unit in 4 cells and had no measurable effect in 4 others. These results suggest that Na/H exchange plays no role in the reduction of pH, in response to extracellular acidosis. Furthermore, HOE-642 did not alter spontaneous electrical activity, did not prevent the changes in electrical activity induced by pH 6.6 ischemic Tyrode, and did not prevent recovery of electrical activity after washout of pH 6.6 ischemic Tyrode solution. Thus we conclude that Na/H exchange does not contribute to these changes nor does it facilitate recovery from such effects.

Ionic mechanisms. The results of previous investigations can suggest which currents might have been altered to produce the observed changes in spontaneous electrical activity. For example, reducing pH, to 6.5 decreased the rapid component of IK (IK,r), whereas increasing pH, to 8.5 enhanced IK,r in rabbit ventricular myocytes (42). In SA node pacemaker cells, IK was reduced by cyanide (18) or by acidosis (pH, = 6.5) (37), and partial blockade of IK,r by E-4031 depolarized the MDP and reduced the OS (43). These results suggest that in the present study, depolarization of the MDP by pH 6.6 ischemic Tyrode solution or by pH 6.8 Tyrode solution, and hyperpolarization of the MDP by pH 8.5 Tyrode solution could have been due to reduction and enhancement of IK,r, respectively. Cyanide (18) and acidosis (pH, = 6.5; Ref. 37) also reduced ICa,L in SA node pacemaker cells, and such a reduction could have attenuated IK and thereby depolarized the MDP if the peak of the AP were reduced sufficiently. Although we cannot rule out a direct effect of acidosis on the L-type Ca single-channel conductance, inactivation of ICa,L by a depolarized membrane potential is sufficient to explain the reduction of OS in the present study, because even in the presence of pH 6.6 ischemic Tyrode solution, injection of hyperpolarizing current and the consequent recovery of MDP to a more negative potential returned the OS to its original level. On the other hand, the OS did not increase when pacemaker cells were exposed to pH 8.5 Tyrode solution and the MDP hyperpolarized. Nevertheless, this result might be explained by the fact that the original MDP (−64 ± 2 mV) was already sufficiently negative to remove any

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inactivation of $I_{Ca,L}$. (15). Hyperpolarization of the MDP when pacemaker cells were exposed to pH 8.5 Tyrode suggested that we would see a similar effect when NH$_4$Cl increased pH; however, the MDP depolarized significantly during this period. Such a depolarization, which has been observed previously under similar conditions (6, 41), has been attributed to the reduced electrochemical gradient for $I_K$ when NH$_4$Cl-containing Tyrode solution is first introduced because NH$_4^+$ is permeable through K channels (6).

Finally, our voltage-clamp recordings suggest that even though ischemic conditions have no significant effect on $I_T$, they do enhance time-independent $I_{initial}$ in the pacemaker range of potentials (~70 to ~60 mV). This increase is more likely to result from reduction of an outward current than from enhancement of an inward current, because acidic pH reduces the single-channel conductance and open probability of most ion channels (9). Therefore, acidosis-induced enhancement of sustained inward current (17) or TTX-sensitive “window” current (30) is unlikely. Because 30 µM BaCl$_2$ in normal Tyrode solution increased $I_{initial}$ in 4 pacemaker cells and because concentrations ~50 µM are selective blockers of inward-rectifying K currents in ventricular myocytes (21) and cardiac Purkinje fibers (11, 40), we speculate that BaCl$_2$ blocked an inward-rectifying K current in our experiments. Although we have no information on the identity of this current, we speculate that it might be ACh-activated K current ($I_{K,ACH}$), because this current is activated in rabbit SA node pacemaker cells even in the absence of ACh (23), whereas $I_K$ is rarely found in these cells (32). Nevertheless, the actual identity of the ischemia-sensitive current is unknown. Therefore, additional voltage-clamp measurements of time-dependent and -independent currents, fluorescence measurements of Ca$_i$, and models of isolated SA node pacemaker cells (e.g., Ref. 13) will be necessary to fully understand the mechanisms responsible for the ischemia-induced changes.

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Present address of J. Qu: Dept. of Pharmacology, Columbia University, College of Physicians and Surgeons, New York, NY 10032.

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